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13. ABSTRACT (Maximum 200 words)  The aims of this research were to study each of the various molecular mechanisms whereby toxic metal cations and oxyanions were chemically transformed by <u>Pseudomonas maltophilia</u> strain OR02. The research effort focused on the microbial-dependent transformations of mercury, selenium, tellurium, chromium, lead, cadmium, silver, and gold. The NADPH-dependent reduction of Hg(II) was catalyzed by an inducible mercuric reductase. The reduction of selenite and tellurite to their insoluble elemental forms was mediated by an intracellular glutathione reductase that utilized the spontaneously-formed bis(glutathio)Se or bis(glutathio)Te, respectively, as pseudosubstrates. The 3-electron reduction of hexavalent chromium was catalyzed by a membrane-bound chromate reductase. The enzymatic basis for the transformation and immobilization of soluble lead(II), cadmium(II), silver(I), and gold(III) was not immediately apparent. This project could provide useful information toward the eventual exploitation of <u>P. maltophilia</u> and related organisms for the removal of toxic metal wastes from selected, heavily polluted sites.			
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## FINAL TECHNICAL REPORT

May 1, 1989 - April 30, 1992

AFOSR Contract F49620-89-C-0052: Transformation and Precipitation of Toxic Metals by Pseudomonas maltophilia

### OVERVIEW

The overall aims of this project were to study each of the various molecular mechanisms whereby toxic metal cations and oxyanions were chemically transformed by a remarkable strain of Pseudomonas maltophilia originally isolated from mercury-contaminated soil at Oak Ridge National Laboratory. The specific aims for the previous grant period were as follows:

- (1) To perform detailed kinetic studies on selected metal transformations using suspensions of intact bacterial cells;
- (2) To determine whether each metal transformation is a function of the bacterial cell itself or some exported component(s); and
- (3) To identify, separate, purify, and reconstitute the minimum cellular components necessary for metal transformation.

The metal cations and oxyanions examined in these investigations included, but were not limited to, Se(IV), Cr(VI), Pb(II), Ag(I), Au(III), Cd(II), and Hg(II). Experimental results that addressed each specific aim are summarized below.

### RESEARCH ACCOMPLISHMENTS

#### Kinetic studies with intact bacterial cells

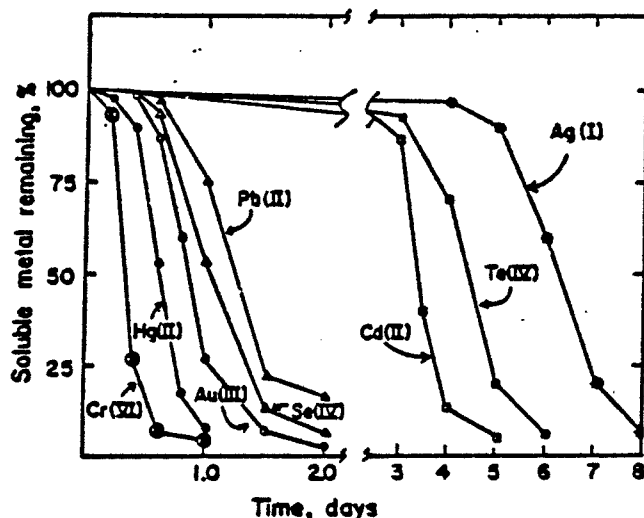
When P. maltophilia strain OR02 was cultured in the presence of each of 8 different soluble metal species, growth of the organism was accompanied by the disappearance of the soluble metal species from solution, along with the concomitant appearance of an insoluble form of the metal. The kinetic properties of the P. maltophilia-dependent removal of soluble metals are shown in Fig. 1. Each curve in Fig. 1 was generated by the introduction of naive, unadapted bacteria to the culture vessel containing the soluble metal species. Initial soluble metal concentrations in Fig. 1 were as follows: Cr(VI), 1.0 mM; Hg(II), 0.2 mM; Au(III), 3.0 mM; Se(IV), 40 mM; Pb(II), 3.0 mM; Cd(II), 3.0 mM; Te(IV), 10 mM; and Ag(I), 4.0 mM. The disappearances of 5 of the soluble metals in Fig. 1 were linked to biological reduction reactions. Selenite, tellurite, mercuric ions, silver ions and chloroauric acid were each reduced to their respective elemental forms.

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Fig. 1. Time courses of metal disappearance



The bacterial-dependent reduction of hexavalent chromium resulted in the transient formation of trivalent chromium, which subsequently disappeared from solution concomitantly with the formation of a white precipitate. Growth of strain OR02 in the presence of lead and cadmium resulted in the formation of brown-black and white precipitates, respectively, along with the concomitant removal of each soluble cation from solution.

Efforts to quantify the soluble metals featured in Fig. 1 were limited to UV-visible spectrophotometric assays that employed colorimetric reagents whose molecular absorption properties changed in the presence of specific metal ions. The practical detection limits of such assays are no lower than about 1.0 micromolar at best. With the acquisition of a combination flame/graphite furnace atomic absorption spectrophotometer (funded by the AFOSR) about 30 months into the project period, it became possible to lower the operational detection limit for most soluble metals shown in Fig. 1 by some 2-3 orders of magnitude. An investigation of the kinetic properties of metal removal by strain OR02 at much lower concentrations of soluble metal was initiated and is still in progress. Thus, strain OR02 was demonstrated to scrub solutions of Hg(II), Se(IV), Pb(II), Ag(I), and Cd(II) to 0.1, 0.5, 1.0, 10, and 160 micrograms/liter, respectively. While cell growth and metal-transformation activities at the high metal concentrations shown in Fig. 1 are unexpected and dramatic, it is the performance of metal-immobilization microbes at the lower concentrations more frequently encountered in polluted waste waters that is of the greater practical significance.

A manuscript that describes these results entitled "Chemical transformation of toxic metals by a *Pseudomonas* strain from a toxic waste site" has been accepted pending mandatory revisions in the journal of Environmental Toxicology and Chemistry.

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### Cellular location of metal transformation

The location, either intracellular or extracellular, of each metal precipitation activity attributed to strain OR02 was determined by scanning electron microscopic (SEM) and energy dispersive X-ray (EDX) analyses performed in collaboration with Drs. Larry Barton and Thomas Zocco at New Mexico State University and Los Alamos National Laboratory, respectively. Examination by SEM of cultures of OR02 grown in the presence of 40 mM selenite revealed 3 principal features: (i) intact bacterial cells frequently contained one or more electron dense bodies; (ii) other electron dense bodies were observed outside the cells; and (iii) numerous lysed cells and cell fragments were evident. EDX analyses on the same samples revealed that the electron dense bodies both inside and outside the cells were comprised exclusively of selenium and that these bodies accounted for all of the selenium in the field of vision. When OR02 was cultured in the presence of much lower concentrations of selenite, such as 1.0 mM or less, the number of both the extracellular selenium deposits and the lysed cells decreased dramatically relative to the number of cells with intracellular selenium deposits. On the hypothesis that the extracellular selenium deposits arose as a consequence of the lysis of bacterial cells at high selenite concentrations, the SEM and EDX analyses suggested that elemental  $\text{Se}^0$  was produced intracellularly.

In contrast to the results obtained with selenite, growth of strain OR02 in the presence of lead nitrate led to the appearance of dark, electron dense bodies outside the bacterial cells. These dark extracellular bodies contained all of the detectable lead; lead could not be detected by EDX spectroscopy either inside the cell or on the plasma membrane. The SEM and EDX analyses thus indicated that insoluble lead was generated extracellularly.

Table I. SEM/EDX analyses of metal precipitation by strain O-2

Soluble metal species	Intracellular ppt.	Extracellular ppt.
Se(IV)	X	
Te(IV)	X	
Hg(II)	X	
Au(III)		X
Ag(I)		X
Pb(II)		X
Cd(II)	Could not be determined	

Experiments such as those outlined above were conducted on each of 7 soluble metal species transformed and precipitated by strain OR02. The conclusions regarding the site(s) of metal transformation by strain OR02 are summarized in Table I, above.

Identification, separation, purification, and reconstitution of the minimum cellular components necessary for metal transformation

A. Mercury - A mercuric reductase was purified to electrophoretic homogeneity from cell-free extracts of OR02 grown in the presence of 100  $\mu\text{M}$  Hg(II). The purified enzyme was a soluble dimer comprised of identical subunits of 60,000 daltons. The enzyme contained one FAD per subunit and catalyzed the NADPH-dependent reduction of Hg(II) to Hg(0) in the presence of excess exogenous thiols. With 2-mercaptoethanol as the exogenous thiol, the reduction of mercuric ions obeyed Michaelis-Menten saturation kinetics with values of  $K_m$  for NADPH and RSHgSR of 15 and 6.0  $\mu\text{M}$ , respectively, and a turnover number of 270  $\text{min}^{-1}$ . The structural and functional properties of the mercuric reductase from OR02 were thus similar to those of analogous enzymes from other bacteria. The mercuric reductase-dependent oxidation of NADPH was entirely specific for mercuric ions. No enzyme-dependent oxidation of NADPH could be detected in the presence of any of the other 7 soluble metal species in Fig. 1. These experimental results are presented in the manuscript submitted to Environmental Toxicology and Chemistry.

B. Selenium - Experimental results with both whole cells and cell-free extracts indicated that the bacterial-dependent generation of elemental selenium occurred as a consequence of the glutathione reductase-dependent reduction of the bis(glutathio)Se that forms spontaneously when selenite is exposed to a molar excess of reduced glutathione in the cytoplasm of the bacterial cell.

A manuscript that describes these experiments with selenite entitled "On the microbial-dependent transformation of toxic metals: mechanism of selenite reduction by Pseudomonas maltophilia" was favorably received by the Journal of Biological Chemistry and is currently undergoing minor revisions.

C. Tellurium - The current working hypothesis is that the bacterial-dependent generation of elemental tellurium is strictly analogous to that of elemental selenium. That is, the reduction of tellurite to tellurium occurs as a consequence of the glutathione reductase-dependent reduction of the bis(glutathio)Te that forms spontaneously when tellurite is exposed to a molar excess of reduced glutathione in the cytoplasm of the bacterial cell. Rapid mixing spectrophotometric experiments to document and characterize the abiotic formation of bis(glutathio)Te are currently in progress. It is anticipated that these experiments will eventually lead to a separate publication.

D. Chromium - Cells of strain OR02 that had been adapted for the reduction of chromate to chromium(III) were disrupted and examined for a cell-free chromate reductase activity. The only cell-free, pyridine nucleotide-dependent reduction of chromate that could be detected was located in the membrane fraction of cells that had been disrupted by sonic oscillation. Both NADH and NADPH supported chromate reduction. The pH optimum was 7.5 with an apparent  $K_m$  for chromate of 100  $\mu$ M. The membrane-bound chromate reductase activity was quite labile and lost 70-80% of its original activity after 24 hours at 4° C. The enzyme(s) responsible for chromate reduction have not been investigated further because of this discouraging stability problem.

E. Lead - Efforts to identify the chemical nature of the black precipitate generated when OR02 was grown in the presence of Pb(II) were inconclusive. Samples of the black precipitate were collected and washed exhaustively with hot sodium dodecyl sulfate to remove cellular materials. A washed specimen was subsequently submitted to Surface Science Laboratories, Mountain View, CA, for ESCA analysis (Electron Spectroscopy for Chemical Analysis). The ESCA spectra showed unequivocally that the lead in the black precipitate was not elemental lead. Instead, the lead was cationic, either Pb(II) or Pb(IV). These experiments are described in the manuscript submitted to Environmental Toxicology and Chemistry. Whatever the cell produces to coordinate this cationic lead, it complexes the lead much more tightly than does EDTA (since soluble Pb(II) coordinated to EDTA was also rendered insoluble by cells of OR02). Chemical analysis of this black precipitate will continue.

In the meantime, we discovered that the OR02 appears to express an organolead lyase activity. When grown in the presence of up to 1.0 mM mono-, di-, or triethyllead chloride, OR02 generated the same black precipitate as that obtained in the presence of mere inorganic Pb(II). Such an organolead lyase activity has never been described in the literature. We intend to further examine the apparent substrate specificity of this organolead lyase using whole cells and then attempt to purify the activity from cell-free extracts of the organism.

Bacteria from other toxic waste sites catalyze metal transformations similar to those of strain OR02.

A collaboration was initiated with the laboratory of Dr. Larry Barton at New Mexico State University in Albuquerque, NM. In addition to the SEM experiments discussed above, we have participated in the isolation and preliminary characterization of metal-transforming bacteria from chromium(principally chromate)-contaminated soil at Sandia and Los Alamos National Laboratories. Several isolates have been discovered that electrochemically reduce chromate and mercuric ions. In addition, a handful of these isolates also transform soluble lead in a manner similar to that of strain OR02.

A manuscript that describes these preliminary experiments entitled "Application of biotechnology in management of industrial wastes containing toxic metals" was submitted to Radioactive Waste Management and the Nuclear Fuel Cycle Journal.

Acquisition of these new isolates creates the possibility of investigating particular metal-transformation activities in more than one organism. The knowledge accumulated within each stable of organisms that appear to share a fundamentally common pathway of metal transformation may provide opportunities to study a particular portion of the pathway in some of the members that proves to be intractable in other members. For example, a key protein in the pathway that is extremely difficult to isolate from one organism may prove to be easily obtained in good yield from another. On the other hand, detailed comparisons among the groups of organisms that appear to express different mechanisms and pathways for selected metal transformations may provide the opportunity to deduce the advantages and disadvantages of each. In terms of the eventual application of this basic knowledge to bioremediation problems, it should be emphasized that each metal-transforming organism was isolated from a hazardous waste environment. Although strain OR02 has received the most attention in the laboratory, that does not mean that it will prove to be the predominant or most useful organism in any or all of the eventual bioremediation processes that may be developed. The need for basic information regarding the metal transformation activities of all of these organisms is evident.

Some of the metal transformation activities of strain OR02 have been mobilized to other bacteria.

A second collaboration was initiated between this laboratory and that of Dr. Julius Jackson, a molecular geneticist at Michigan State University. Dr. Jackson used plasmid DNA derived from strain OR02 to transform various metal-resistance phenotypes into a recipient strain of E. coli. He has supplied this laboratory with stable E. coli transformants that either (i) reduce Se(IV) to elemental selenium, (ii) reduce Hg(II) to elemental mercury, or (iii) immobilize Pb(II) as a brown-black precipitate. The acquisition of these transformants broadens the opportunities to study the molecular mechanism(s) of each metal transformation. The ability to transfer metal-immobilization phenotypes into other bacteria could also permit the eventual transformation of indigenous bacterial populations already adapted to and inhabiting selected heavily polluted sites.

As a result of these promising preliminary experiments, this laboratory recently attracted 2 graduate students who wish to pursue their thesis research on molecular aspects of the bacterial-dependent transformation of soluble metals. One of these students will be funded by an Air Force AASERT grant.

Certain facultative anaerobic bacteria will electrochemically reduce insoluble metal oxides.

A third collaboration was initiated with Dr. Patricia Rusin, senior microbiologist with Metallurgical and Biological Extractions, Inc., Tucson, AZ. Dr. Rusin isolated over 300 bacteria from Crystal Hill, CA, and Hardshell, AZ, mine samples for their ability to electrochemically reduce and solubilize manganese dioxide under anaerobic conditions. Isolate D1 demonstrated a capacity to reduce manganese dioxide superior to that of any other organism described to date. Furthermore, strain D1 also exhibited the facile electrochemical reduction of insoluble iron oxides. This bacterium and others like it hold great promise for the bioremediation of a variety of insoluble metal oxides. Indeed, at the PI's suggestion, Dr. Rusin spent 2 weeks doing collaborative experiments with Dr. James Brainard at Los Alamos National Laboratory in New Mexico. They were able to demonstrate the facile bacterial-dependent electrochemical reduction of insoluble plutonium dioxide [Pu(IV)] to soluble Pu(III). It may thus be possible to devise strategies to remediate plutonium-containing materials based on the bacterial-dependent mobilization of the insoluble radionuclide into the aqueous phase. This laboratory will investigate the biomolecules responsible for this remarkable electrochemical reduction reaction.

#### PUBLICATIONS

(i) Published - none

(ii) Submitted - three

"Chemical transformation of toxic metals by a Pseudomonas strain from a toxic waste site"; R.C. Blake, D.M. Choate, S.H. Bardhan, N.H. Revis, L.L. Barton, and T.G. Zocco; submitted to Environmental Toxicology and Chemistry

"On the microbial-dependent transformation of toxic metals: mechanism of selenite reduction by Pseudomonas maltophilia"; R.C. Blake, D.M. Choate, S.H. Bardhan, N.H. Revis, and J.H. Jackson; submitted to the Journal of Biological Chemistry

"Application of biotechnology in management of industrial wastes containing toxic metals"; L.L. Barton, F.A. Fekete, L.O. Sillerud, C.J. Pigg, and R.C. Blake; submitted to Radioactive Waste Management and the Nuclear Fuel Cycle Journal

(iii) In preparation - none

#### PROFESSIONAL PERSONNEL

(i) Postdoctoral Associate - None

(ii) Research Assistant - Donna Choate, employed for the

last 31 months

#### COUPLING ACTIVITIES

(i) Meeting presentations - two, "Chemical transformation of toxic metals by a Pseudomonas strain from a toxic waste site"; D. Choate, R.C. Blake, R. Revis; presented at the 1991 Annual Meeting of the American Society for Biochemistry and Molecular Biology held at Atlanta, GA; and

"Chemical transformation of toxic metals by a Pseudomonas strain from a toxic waste site"; R.C. Blake, D. Choate, and N.R. Revis; presented at the 11th Annual Meeting of the Society for Environmental Toxicology and Chemistry held at Washington, D.C.

(ii) Consultations - five invited seminars at the following institutions: New Mexico State University, Albuquerque, NM; Montana State University, Bozeman, MT; Los Alamos National Laboratory, Los Alamos, NM; Clemson University, Clemson, SC; and Chrysol, Inc., Tucson, AZ.

#### NEW DISCOVERIES

That strain OR02 appears to contain an organolead lyase activity

That selected metal-transformation activities are plasmid-borne and may be mobilized into other bacteria